COMPARATIVE GENOMIC ANALYSIS OF *PANTOEA ANANATIS* NCCP568 TO STUDY ITS GENOMIC DIVERSITY AND ANTIBIOTIC RESISTANCE

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Science

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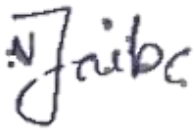
Signature of student…  .

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Noor Zainab

Dedicated to my son

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List of abbreviations

AMR	Anti- Microbial Resistance
BPGA	Bacterial Pan Genome Analysis
CARD	Comprehensive Antibiotic Resistance Database
CDS	Protein coding sequences
CSLI	Clinical and Laboratory Standards Institute
ICEs	Integrative and Conjugative Elements
INA	Ice Nucleation Active gene
MGE	Mobile Genetic Elements
MSA	Mannitol Salt agar
NARC	National Agriculture and Research Center
NCCP	National Culture Collection of Pakistan
PA	*Pantoea ananatis*
RGI	Resistance Gene Identifier

Abstract

Pantoea ananatis is gram-negative bacteria of rod-shape, facultative anaerobic nature and member of the Erwiniaceae family of the Enterobacteriaceae group. In addition to occurring endophytically in hosts where it has been documented to induce signs of disease as well as in species where no such symptoms have been detected, it is mostly epiphytic in nature. Its Ubiquitous nature makes it biotechnologically and environmentally significant. In order to better understand the genomic diversity and antibiotic resistance a local *P. ananatis* strain NCCP 568 was sequenced using universal 16S rRNA sequencing primers at Macrogen, Korea (Seoul, Korea). The genomic size of the strain NCCP 568 is 4.685 Mbp with 53.49% GC content, 0 plasmids and 21 contigs. The genome contains 4764 protein coding genes and 68 non-coding RNA genes. The closely related *P. ananatis* strains include PA13 which is representative strain from Korea, JT1-188, R100, JT8-6, YJ76, NN08200, JBR-LB3-16, FDAARGOS_680, AJ13355, LMG 5342 and TZ39. Several resistant genes were identified in *P. ananatis* NCCP568 including vanG, ArnT, PBP3, gyrB, CRP, rsmA, KpnH and adeF genes. These genes are known for conferring resistance aagainst vancomycin, colistin, beta-lactam antibiotics, fluoroquinolones, erythromycin, cloxacillin, oxacillin, norfloxacin, azithromycin, imipenem, polymyxin and tetracycline. Comparative analysis of NCCP568 strain with other complete genome sequence strains revealed that the local strain NCCP568 is more resistant and virulent compare to other complete genomes. Phylogenetic analyses inferred that NCCP568 is closely related to FDAARGOS, JT1-188, TZ39, JT8-6 and Lstri and distantly related to PA13 reference strain.

CHAPTER 1

1. Introduction

1.1 Background of *P. ananatis*

Pantoea ananatis is a species of gram-negative, rod-shaped bacteria that previously belonged to the family Enterobacteriaceae and was later added to the family Erwiniaceae (De Maayer et al., 2014). It was first identified and named in 1990 (Weller-Stuart et al., 2017). *P. ananatis* is commonly found in a variety of environments, including soil, water, plants, and animals. It is known for its plant-associated lifestyle and can be found as a commensal or a pathogen in several plant species. *P. ananatis* has a rod-shaped morphology and is facultatively anaerobic, which means it can survive and grow both in the presence and absence of oxygen (De Maayer et al., 2014). It is motile due to the presence of flagella, which allows it to move in liquid environments (Lv et al., 2022). As a plant pathogen, *P. ananatis* has been associated with various diseases and infections in a wide range of economically important plants, including maize, onion, rice, pineapple, and eucalyptus. It can cause symptoms such as leaf spot, blight, stem rot, and soft rot, leading to reduced crop yield and economic losses in agriculture (Morin, 2014). However, not all strains of *P. ananatis* are harmful to plants. Some strains have been found to promote plant growth and offer protection against certain plant pathogens (Cooney et al., 2014). These beneficial strains have the ability to produce plant growth-promoting substances and induce systemic resistance in plants, enhancing their tolerance to various stresses(De Maayer et al., 2014). In addition to its plant association, *P. ananatis* has also been isolated from clinical specimens, such as blood, urine, and respiratory samples, in rare cases of human infections. However, it is generally considered an opportunistic pathogen and is not a common cause of human disease (Lu et al., 2021).

The genetic analysis of *P. ananatis* plays a crucial role in understanding its biology, pathogenicity, and potential applications in the environment and biotechnology. Genetic studies help elucidate the virulence factors and mechanisms employed by *Pantoea ananatis* researchers can gain insights into the molecular interactions between the bacterium and its host, ultimately leading to the development of effective disease management strategies (Yu et al., 2022). *P. ananatis* exhibits potential for biotechnological applications. Genetic studies can uncover genes responsible for the production of valuable secondary metabolites, enzymes, and other bioactive compounds. These genetic insights can aid in optimizing production processes, improving yields, and enhancing the efficiency of biotechnological applications such as enzyme production, biocatalysis, and pharmaceutical development (Shin et al., 2023). Understanding the genetic makeup of *P. ananatis* enables researchers to manipulate its genome and engineer strains with desired traits. This opens up opportunities for synthetic biology approaches, including the introduction of novel metabolic pathways, the enhancement of desired traits, and the development of genetically modified strains for specific purposes(Luna et al., 2023). Some strains of *P. ananatis* have shown the ability to degrade various environmental pollutants, including hydrocarbons and heavy metals. Genetic studies can help identify the genes and enzymes involved in these degradation processes, allowing for the development of improved bioremediation techniques to address pollution issues and restore contaminated environments (Arayaskul et al., 2020).

1.2 Introduction to Comparative Genomics

In the branch of research known as comparative genomics, the genomes of various creatures are compared in order to learn more about their evolutionary links, functional traits, and genetic variants. It involves analyzing and comparing the DNA sequences, gene arrangements, and other genomic features across multiple species or individuals (Toh et al., 2019). The advent of high-throughput DNA sequencing technologies has revolutionized

comparative genomics, allowing researchers to sequence and compare the entire genomes of various organisms more efficiently and at a lower cost. Comparative genomics helps in understanding the evolutionary history and relatedness of different species. By comparing the genomes of different organisms, scientists can infer common ancestry and track evolutionary changes. It provides insights into the processes of speciation, adaptation, and the emergence of new traits (Toh et al., 2019). Comparative genomics aids in identifying and characterizing functional elements within genomes, such as protein-coding genes, non-coding RNAs, regulatory regions, and conserved DNA sequences (Abdel-Gaied et al., 2022). By comparing genomes across species, researchers can determine which regions are conserved and likely to play important roles in the organism's biology, such as genes involved in basic cellular functions or genes associated with specific traits or diseases. Comparative genomics allows the study of gene gain, loss, and rearrangements across species. It helps identify genes that have been conserved throughout evolution and those that are unique to certain lineages. Comparative analysis can provide insights into the processes of gene duplication, gene loss, and the evolution of novel gene functions (Peng et al., 2023).

1.3 Objectives

The main objectives of this study were the following:

1. To study the genomic diversity of *P. ananatis* NCCP 568 through comparative genomic analysis.
2. To study the antibiotic resistance of NCCP 568 using RGI CARD.

(Resistance Gene Identifier RGI tools of Comprehensive Antibiotic Resistance Database CARD).

CHAPTER 2

2. Literature review

2.1 Taxonomy of *P. ananatis*

The word "Pantoea" is taken from the Greek word "Pantoios" which means "from sources of all sorts". This gives us an idea of the diverse nature of this group and how it can be extracted from different sorts of ecological and geographical niches (Yoshimura et al., 2022). Because of this reason originally the classification of *Pantoea* was confusing and complex. Originally Gavini et al. (1989) classified some of the members of Pantoea as *Bacillus agglomerans* and *Enterobacter agglomerans*, which was later clarified. In 1992, three new strains, which were isolated from soil and fruits, were observed to have similar characteristics as that of family Enterobacteriaceae. Later through DNA hybridization, these species were added to the Pantoea group as *Pantoea punctata*, *Pantoea citrea,* and *Pantoea terrea (Toh et al., 2019).* Later, fatty acid analysis and DNA-related investigations were used to classify *Erwinia uredovora* and *E. ananas* into comparable species, leading to the creation of two new species, *P. ananatis,* and *P. stewartii*. Within *P. stewartii*, two subspecies called *P. stewartii spp. stewartii* were produced. Currently, there are 20 recognized species in the Pantoea genus, which are divided into 13 hybridization groups and share a similar morphological profile (Krawczyk et al., 2021).

2.2 Ubiquitous Nature of *P. ananatis*

P. ananatis is a generic epiphyte that may also grow as an endophyte on both organisms which has been discovered to induce symptoms of disease and hosts where none have been observed. Some strains have the ability to form ice crystals, which is employed by the food

and as a biological control technique against specific insect pests that damage crops (Azizi et al., 2019).

Both antifungal and antibacterial activities are present in *P. ananatis*. Specialists in biological control may be able to take use of these traits (Toaza et al., 2021).

2.2.1 Epiphytic *P. ananatis*

It is an epiphyte that may grow on both hosts and non-hosts. It has also been noted to live as an epiphyte on a number of commercially valuable plant species, including wheat heads, cotton lint, poplar trees, mulberries, and smut of maize (Arayaskul et al., 2020). *P. ananatis* was identified as an epiphyte from a host, although its presence on the surface of plants was not always related to a specific disease on the host. But it's likely that these silent non-hosts are serving as a reservoir of infection for neighbouring vulnerable hosts, causing outbreaks of disease. (Lao et al., 2023).

P. ananatis on plants might be beneficial to some plants. The bacterium exhibits both *in vivo* and *in vitro* antibacterial and antifungal activities, defending host plants from contamination by other dangerous fungi and bacteria (Bajpai et al., 2020). A previously identified strain known as *P. uredovora* was discovered to have in vitro antibacterial activity against all organisms in the "amylovora" group, including *Agrobacterium* (Bomfeti et al., 2008*)*. Strong antifungal activity against *Rhizopus* species has been demonstrated by its isolates from buck weed seed *in vitro*. A *P. ananatis* strain called CPA-3 was found to quickly colonise the scars of harvested pome plants, preserving them from *Penicillium spp*. Similar to this, it has been discovered that certain *P. ananatis* strains may protect tomato fruit from *Botrytis cinerea,* a fungus that causes grey mould (Enya et al., 2007). A strain of *P. ananatis* that has been modified with the *Serratia marcescen's* chitinolytic enzyme gene (ChiA) has also proved successful in controlling *Pyricularia oryzae's* rice blast (Cui et al., 2023).

Only a few bacteria species have been found to include ice-nucleating strains, including *Pantoea ananatis*. InaA, a sequenced ice nucleation active (INA) gene, was discovered to be related to *Pseudomonas* species (Lana et al., 2012). This means that slightly greater temperatures than would typically happen when the bacteria absent are used to promote frost development on sensitive plants. Eucalyptus, pea, palm, and other fruit trees are a few examples of plants that are sensitive to frost. *Pantoea ananatis* is identified as the predominant maize leaf pathogen in China, and it has also been linked to frost injury in mulberries, strawberries, tea, apricots, apricots, citrus, and tea. It is unclear exactly how frost damage contributes to epidemics of many of the conditions brought on by *P. ananatis* (Miller et al., 2016).

2.2.2 Endophytic *P. ananatis*

Even while fungi are more frequently thought of as endophytes, bacteria can also colonize plants. In this setting, they could be deemed advantageous, potential disease-causing agents that are latent or neutral (Escanferla et al., 2018). *P. ananatis* has been identified as an endophyte in several plants in recent years, such as coffee. Its function has primarily been discovered to be advantageous to the plant. *P. ananatis* significantly increases pepper growth and creates total resistance to *Xanthomonas spp*. Similarly, when *P. ananatis* strains were introduced to papaya shoot tips that had previously been endophytic inhabitants, they showed noticeably greater root and shoot growth (Krawczyk et al., 2010). Endophytic stains found in maize kernels exhibited in vitro protection against *Lecanicillium aphanocladii*. Few instances of *P. ananatis* acting as a lethal pathogen within host cells have been documented. It is noted in rice plants as an endophyte. Additionally, the endophytic *P. ananatis* is also found in rice seeds. Both are recognized hosts for this virus, and its presence in seeds may have significant epidemiological implications (Hara et al., 2012).

2.2.3 Saprophytic *P. ananatis*

Pantoea ananatis has been isolated from a diverse range of environments as a saprophyte. These include rivers, soil, aviation fuel tanks, rice fermentation, from the rhizosphere of soft rush and on insects, and is a primal inhabitant of the gut microflora of brown plant hoppers, mulberry, cotton flea insects, and tobacco plants (*Frankliniella fusca*). *P. ananatis* has also been isolated from *Pinus spp.* roots colonized by *ectomycorrhizal* in Australia (De Maayer et al., 2014a). Many of these studies which show that *P. ananatis* is present as a saprophyte involved the analyses of bacterial communities either occurring in or contaminating a spot location. The understanding of the inhabitants of these locations was mostly based on sequencing of the 16S rRNA gene and understanding the similarity of sequences to those present in GENBANK. This introduces an element of caution into the interpretation of these results, and one cannot state with confidence that *P. ananatis* does in fact reside in these locations (Kini et al., 2017).

2.2.4 *P. ananatis* as Pathogen

Host and disease symptoms

Both monocotyledonous and dicotyledonous plants can become infected with *Pantoea ananatis*. The significance of this bacterium as an epiphyte or pathogen is not entirely obvious in the several culture collections where it has also been deposited (Shyntum et al., 2015). These hosts include wheat, rice, and sugarcane, all of which have great economic importance to an agricultural country such as Pakistan (Azizi et al., 2019). The host range of the bacteria is depicted in table 1. Depending on the host affected, *P. ananatis* can cause a variety of symptoms, such as patches and spots of die-back, fruit, boll, bulb rot, and decay. Some of these symptoms can be seen in figure 1 (2023). Brown spot symptoms only appear in infected honeydew melons and cantaloupes after harvest According to reports, the disease starts as dormant field infections that wait till the fruit is ripe before becoming active. Young

Eucalyptus trees or seedlings/cuttings that have been infected with *P. ananatis* experience die-back and wilting of the shoots. The main leaf veins and nearby leaf tissue appear to be the first places where the infection spreads from the petioles (Abdel-Gaied et al., 2022).

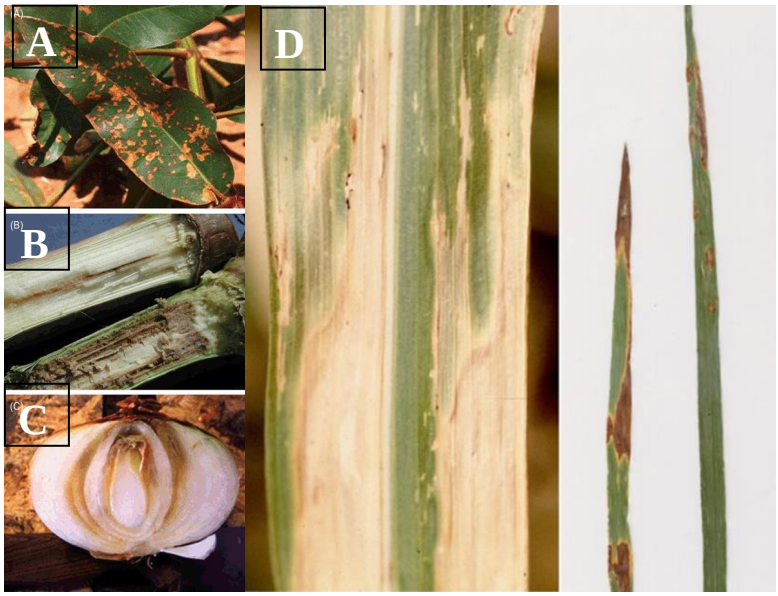

Figure1 : Pathogenic symptoms produced by *Pantoea ananatis*. (A) Bacterial blight in eucalyptus. (B) Brown stalk rot of maize. (C) Center rot of onion. (D) Leaf blight of rice

The indications of infection in monocotyledonous plants include patches and streaks that form corresponding to the main leaf vein. *P. ananatis* is known to produce sporadic disease outbreaks, probably as a result of its opportunistic nature (Shyntum et al., 2015).

The fact that *P. ananatis* symptoms in the same organism might vary from region to region is particularly intriguing (Walterson and Stavrinides, 2015). While stalk rot occurs in South Africa as a result of infection, necrotic or white leaf patches and streaks on maize are identified as the symptoms in Brazil. Similar to how it infects rice in other countries, the pathogen infects the developing seed and makes palea browning; however, in Australia, *P. ananatis* causes stem necrosis as a symptom. NCCP 568 was however collected from a blight-infected rice leaf from Pakistan. This shows that even though the species is the same,

the disease symptoms and expression are different. This gives more reason as to why specific strain from each region needs to be clearly studied and analyzed (Agarwal et al., 2021).

Table 1: *P. ananatis* host range

Hosts	**Natural / Artificial inoculant**	**Symptoms**	**References**
Rice	Natural host	'Palea' browning, Stalk rot, Leaf blight	(Bomfeti et al., 2008)
Sugarcane	Artificial inoculant	Leaf streaks and blotches	(Gu et al., 2022)
Cotton	Artificial inoculant	Internal boll rot, Leaf blight	(Toh et al., 2019)
Maize	Natural host	Spotty Necrosis and streaks, Brown stalk rot	(Mondal et al., 2011)
Pineapple	Natural host	Fruitlet rot	(De Maayer et al., 2017)
Sudan grass	Natural host	Leaf patch and streaks	(Ma et al., 2016)
Cantaloupe fruit	Natural host	Brown spot	(Takumi et al., 2017)
Watermelon	Natural host	Internal boll rot	(Gajdács, 2019)
Onions	Natural host	Blight on leaf, seed stalk rot, decay	(Coutinho and Venter, 2009)
Eucalypts	Natural host	Blight on leaf, shoot tip-back die	(LaGier et al., 2022)
Tomato	Natural host	Graying of wall	(Takumi et al., 2017)
Oyster mushroom	Natural host	Soft rot	(Takumi et al., 2017)

2.2.4.1 Epidemiology

It is not well understood how *P. ananatis* affects various hosts and causes plant diseases. It has been determined that the disease gets into its host plant through blooms, insect-feeding wounds, mechanical damage, and plant-to-plant contact during strong winds. It was discovered that the presence of *P. ananatis* on the leaf surfaces of rice increased the onset of brown hopper burn symptoms (Agarwal et al., 2021). *P. ananatis*, the tobacco trips vector, has been discovered in onion crops. The specific function of *P. ananatis* in disease outbreaks

is presently unknown, despite the fact that it was identified from feeding on diseased plant tissue in a field epidemic of blight on the leaf and back die of eucalypts in South Africa (Takumi et al., 2017). Onions and rice both have the seed-borne and seed-transmitted pathogen *Pantoea ananatis*. This bacterium was also discovered in the kernels of maize and buckwheat, together with many other bacterial species. Seed transmission is suggested by the recent occurrence of bacterial blight and back-die on maize in nations that have imported seed from Africa. Similar to this, outbreaks of onion core rot may be brought about by introducing contaminated seeds into unfamiliar settings or nations (Goszczynska et al., 2006).

The intensity of the infections contracted by *P. ananatis* from its various hosts is influenced by environmental conditions. High humidity and temperate temperatures make the illness more prevalent and severe in maize and eucalyptus, respectively (Krawczyk et al., 2010). Similar circumstances occurred with maize, where *P. ananatis* was discovered to be active during bulb development when water levels were high and temperatures varied between 29 and 35 °C (Cooney et al., 2014).

2.2.4.2 Pathogenesis

According to reports, *Pantoea ananatis* produces indole-3-acetic acid, which may contribute to disease. This plant growth promoter encourages cell wall thinning during cell elongation and has an impact on plants at extremely low doses (Abdel-Gaied et al., 2022). However, it is presently uncertain what *P. ananatis'* main virulence factors are. Recently, the genomes of the type strain from pineapple and a virulent strain of *P. ananatis* from eucalyptus were sequenced using 464 pyrosequencing (De Maayer et al., 2014a).

Initial comparative genomics analysis showed that *P. ananatis* lacks the Type I, Type II, and Type III secretion systems. Numerous bacteria that are linked to animals and other plants have these secretion systems on their pathogenicity islands (Krawczyk et al., 2010). The

unique Type VI secretion system is represented by a group of genes with significant similarity in the *P. ananatis* genome. Several human, animal, and plant-pathogenic Gram-negative bacteria cause illnesses in this system, although its function is still not entirely understood. Signal molecules related to quorum sensing are produced by *Pantoea ananatis*. The N-acyl-Lhomoserine lactones (AHLs) that bacteria created while living in wheat heads include N-hexenoyl-L-hetrmoserine lactone (C7-HSL) and N-(4-oxohexanoyl)-L-hotroserine lactone (4-oxo-C6-HSL). According to Pomini et al. (2006), *P. ananatis* generated three AHLs, with C6-HSL being the main compound.

2.2.4.3 Disease Control

Utilising resistant/tolerant cultivars or clones is often how conditions brought on by *P. ananatis* are controlled. Currently, resistant clones are being used in South Africa to prevent eucalyptus blight and die-back (Morohoshi et al., 2007). They are chosen in a cutting operation nursery with a high disease incidence. Similar to this, it was shown that it was able to choose genome resistant to *P. ananatis* by intentionally inoculating maize lines. Application of the fungicide Mancozeb at the early stages of disease development has also proved successful in controlling the white blotch disease of sorghum in Brazil (Agarwal et al., 2021). To prevent onion core rot, the usage of mulch and systems for irrigation has been researched. The kind of irrigation has little impact on the prevalence and seriousness of illness (Morohoshi et al., 2007). However, compared to the usage of black plastic, using straw as mulch or bare soil was observed to postpone the onset of symptoms by 7–14 days. The best management tactics against *P. ananatis*- and the majority of other phytopathogenic bacterial-caused illnesses are probably prevention and elimination of the original inoculum (Bajpai et al., 2020).

2.3 Pan and Core Genes

The whole set of genes in a number of different organisms is known as the pan-genome. It is the combination of every gene family and unique gene found in every strain (Kido et al., 2008). It contains the junction of gene families, which makes up the core genome shared by all species. Pan genes contain both core genes and accessory genes. Three gene sets—Core, Shell, and Cloud genome—can be found in the pan-genome (Carr et al., 2010). The genes found in every genome examined make up the Core genome. Some publications take the soft-core (>95% occurrence) into consideration to avoid excluding families because of sequencing artifacts. The genes that are present in most genomes (10–95% of the time) make up the Shell genome. Dispensable or Cloud genomes are gene families with less than 10% incidence in more than one genome (Azizi et al., 2019).

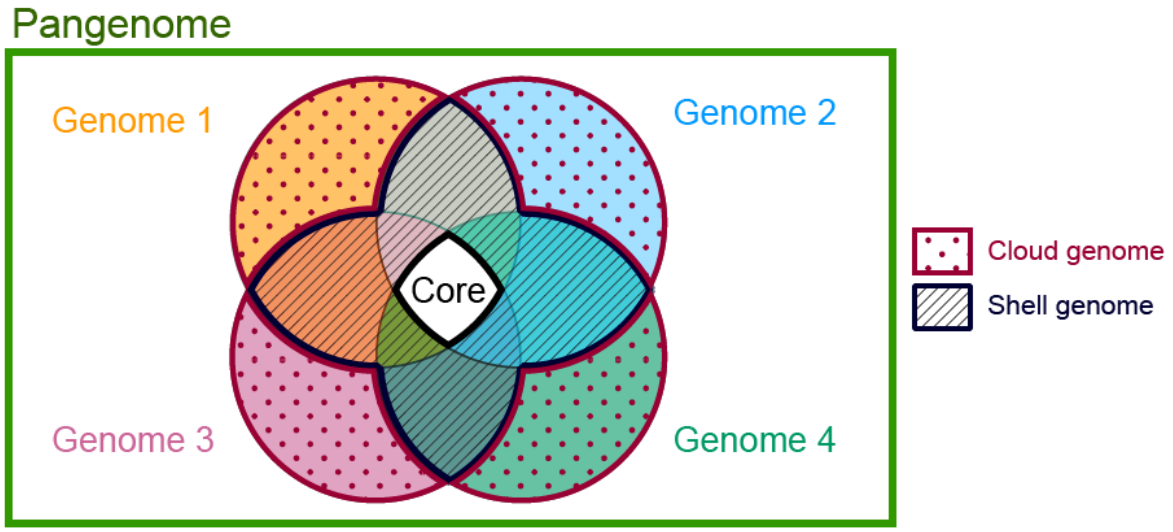

Figure 2: Understanding pan, core, and accessory genes (Carr et al., 2010)

2.4 Pan Genome

The 'open' pan-genome of *P. ananatis* is characteristic of microbial species that colonise a variety of habitats. The pan-genome contains many genes that encode proteins that might help *P. ananatis* colonise, survive in, and perhaps induce disease symptoms in a variety of both animal and plant hosts (Arayaskul et al., 2020).

Recently, the microbial pan-genome has been identified. The pan-genome includes the unique, dispensable, and auxiliary genomes in addition to the core genome (Carr et al., 2010). The core genome is made up of genes that are present in all strains, but the accessory genome, dispensable genome, and unique genome each include genes that are only found in their respective genomes (Azizi et al., 2019). It is conceivable to have more genes than there are genes in a single genome since the pan genome is made up of the gene pool found in specie. More than 239 entire bacterial genomes are already accessible in public databases thanks to the sequencing of the first free-living organism. The insertion of additional gene sequences makes it impossible to adequately define the bacterial species (Weller-Stuart et al., 2017). Therefore, the current best approximation to explain the changing patterns of species is the pan-genome that includes the whole gene pool of the species. High gene diversity in bacterial genomes results from these processes, which can result in the diversification or replication of existing genes. A distinct method of speciation is shown by the pace of diversification. The most frequent method of acquiring new functionalities is through the transfer of genes from unrelated species. Given how quickly bacteria are changing, it is conceivable that the number of microbial gene pons will grow by several orders of magnitude. The existence of more than a billion genes is hardly shocking (Weller-Stuart et al., 2017).

Table 2: Pan genome statistics of some bacteria depicting 'open'-pan genome

Species	Number of strains	Average number of genome CDSs	Average number of core CDSs	Pan-genome compared strains (# CDS)	Unique CDSs per additional strain	References
P. ananatis	8	4,342	4,033	5,566	106	(Cooney et al., 2014)
Streptococcus agalactiae	8	2,029	2,245	2,700	33	(De Maayer et al., 2014b)
H. influenza	13	1,793	1,461	2,786	40	(Ahmed et al., 2014)
Erwinia amylovora	12	3,819	3,414	5,751	52	(Goszczynska et al., 2006)
E. coli	17	5,020	2,344	13,000	300	(Cooney et al., 2014)

2.5 Phylogenetic Analysis

The first complete genome phylogenetic analysis of multi-drug resistant *P. ananatis* strain was reported in Russia in 2011. Although a disease of rice with a similar name has been documented in surrounding parts of China, this is the first case of *P. ananatis* causing the disease in Russia (Egorova et al., 2015). It was also suggested that these genes may have come from the genetic collections of other species or that certain strains of the species had acquired them by horizontal gene transfer (HGT) (Agarwal et al., 2021). Genome diversity is mostly caused by massive recombination events, and the acquisition of such events has changed the genome of P. ananatis. Recombination events are thought to operate as a driving factor in evolution that leads to positive selection in other bacterial genomes. The core genome's single nucleotide polymorphisms (SNPs) have been proposed to have a key role in the selection of multi-resistant strains and in the development of antibiotic resistance in *P. ananatis* (Yan et al., 2010).

To better understand the relation of *P. ananatis* with related spices and non-pantoea groups consider the phylogenetic tree given in Figure 3 (Coutinho and Venter, 2009). Based on the

gyrD sequences of Pantoea species and their closest phylogenetic neighbours, a maximum likelihood tree was created. After 1000 replicates, bootstrap results are presented as percentages. As an outgroup, *Citrobacter rodentium* was added. LMG 2676 and 2678 from *Puccinia graminis* in the USA and China, respectively; LMG 2668 and 2665 from apple in Hawaii and India, respectively; LMG 20103 and BD 1614 from Eucalyptus in South Africa; LMG 2190 from onion in the USA561, 577, 602, 622, 640, 647; and LMG 2491 and 2192 from maize in Africa (Weller-Stuart et al., 2017).

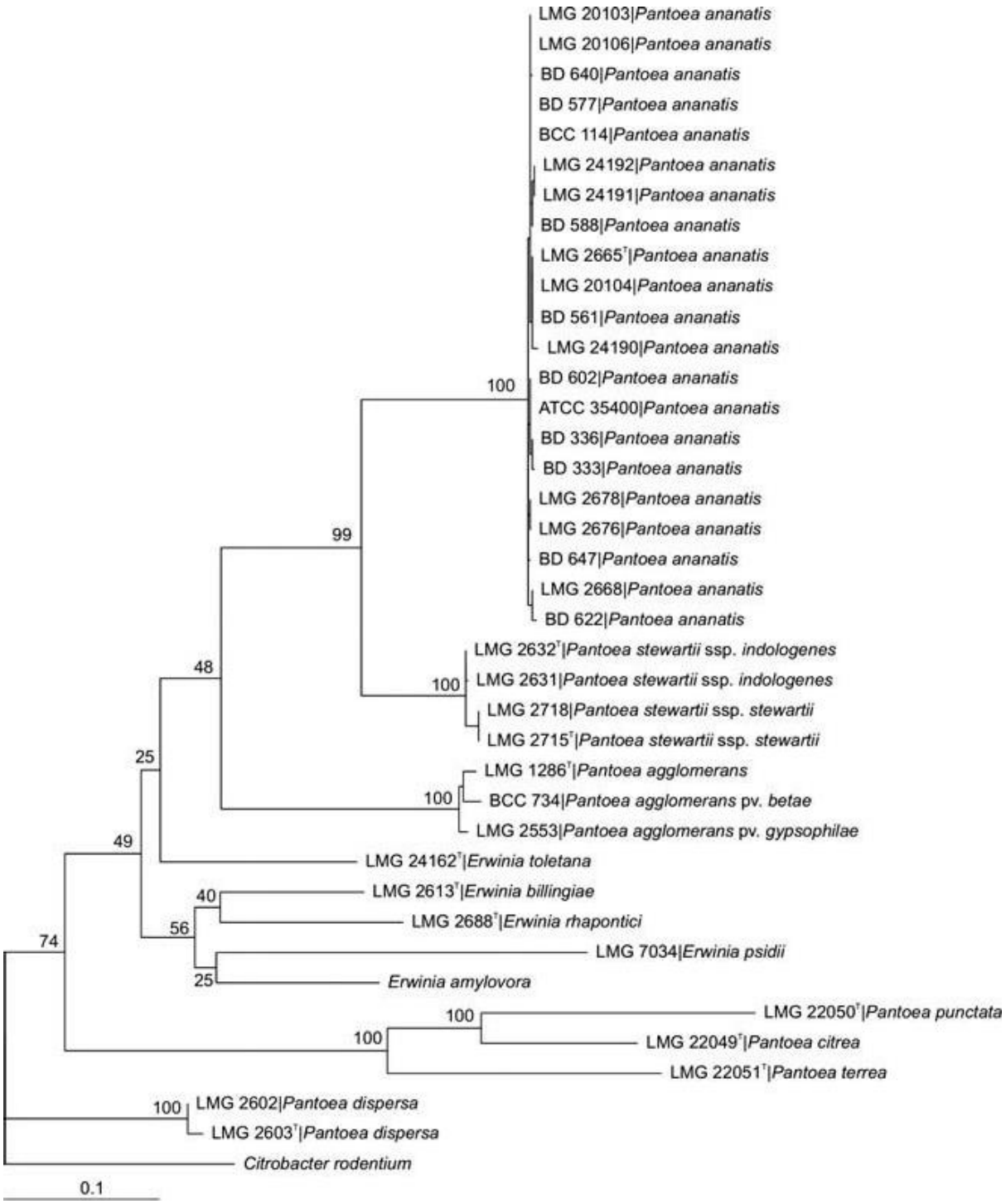

Figure3 : Maximum likelihood phylogenetic tree of *Pantoea spp.* and their phylogenetic neighbours. (Weller-Stuart et al., 2017)

2.6 Antibiotics and mode of action of antibiotics

2.6.1 Inhibition of bacterial cell wall production

The aminoglycosides are regarded as being extremely significant due to their capacity to provide synergistic bactericidal action, especially when combined with antimicrobial drugs like vancomycin that aid in blocking cell wall formation (Stice et al., 2018). Autolysins, enzymes linked to cell walls, are essential for cell growth (Abdel-Gaied et al., 2022). These peptides called AtlE and AtlA, are peptidoglycan (PGN)-hydrolases, which are involved in the destruction of bacterial cell walls. It has also been documented that the N-terminal amidase of AtlE interacts with the pantoea cell wall. Additionally, the presence of autolysins causes the equatorial membrane between both daughter cells to be split and separated (Arayaskul et al., 2020). The mutants with deletions in these important genes showed an unorganized pattern of cell division and were discovered to be biofilm-negative. Reduced cell wall overall turnover is closely correlated with resistance to autolysis (Bomfeti et al., 2008). While certain PGN motifs are more changeable across different species, all bacterial cell walls have common PGN patterns that are predicted by amides (Stice et al., 2018).

2.6.2 Preventing the formation of microbial proteins

The production of protein inhibitor antibiotics, which consist of exotoxins and other key virulence agents in *Pantoea species*, greatly reduces the production of new proteins (Agarwal et al., 2021b). Since ribosomes are essential for protein synthesis, antibiotics frequently target them with the goal of specifically inhibiting bacterial translation by attaching to the bacterial 70S (Hara et al., 2012b). Basically, telithromycin has stronger inhibitory implications for protein synthesis as compared to azithromycin (Bajpai et al., 2020). The peptidyl transferase center (PTC) inside the ribosome is where the oxazolidinones attach to target the bacterial protein synthesis machinery. This inhibits the peptidyl transferase process. Most clinically

proven antibiotic classes, including chloramphenicols, macrolides, lincosamides, streptogramins, and oxazolidinones, prevent protein synthesis by attaching to the peptidyl transferase center (PTC), which is found in the major ribosomal subunit (50S subunit in prokaryotes), which is also known as the peptidyl transferase center. On the basis of biochemical, genetic, and structural investigations, they are clearly known (Stice et al., 2018).

2.6.3 Inhibition of DNA synthesis

Numerous antibiotics that may target RNA polymerase frequently block DNA synthesis or transcription. Most antibiotic molecules, including rifampin, rifapentine, rifaximin, CBR, lipiarmycin, and streptolydigin, have been shown to inhibit transcription in vitro. Their genetic target has also been confirmed by the isolation of resistant mutants in the RNA polymerase genes. The benefits of one antibiotic against other antiseptic agents can be prioritized and taken into account in addition to the physicochemical characteristics of an antibiotic, such as its size or hydrophobicity (Polidore et al., 2021).

2.7 Antibiotic resistance in *P. ananatis*

Through a number of processes that enable it to thrive and multiply in an environment of antibiotics, Pantoea ananatis, like a lot of other bacteria, has the ability to acquire antibiotic resistance. Genetic alterations, horizontal gene transfer, as well as other processes can result in antibiotic resistance. Here are a few methods through which *Pantoea ananatis* might become resistant to antibiotics:

2.7.1 Genetic Mutations: Through unintentional genetic changes, bacteria can become resistant to antibiotics. These mutations can result in modifications to the target regions of antibiotics or changes to metabolic pathways, which reduce the bacterium's susceptibility to the drug's effects.

2.7.2 Efflux Pumps: Efflux pumps are found in some bacteria, such as Pantoea ananatis, and they may effectively transport antibiotics out of a bacterial cell. As a result, the antibiotic is less concentrated inside the cell and hence less effective. Efflux pumps may express themselves normally or become more active in reaction to antibiotic exposure.

2.7.3 Enzymatic Inactivation: It's possible that *Pantoea ananatis* produces enzymes that alter or break down antibiotics, leaving them ineffective. For instance, some bacteria may degrade beta-lactam medicines like cephalosporins and penicillins by producing beta-lactamases.

2.7.4 Target Modification: Antibiotics' target sites can be altered by bacteria, which makes it difficult for the medications to attach correctly and inhibits their intended effects. This may happen as a result of modifications to the target molecule's structure or abundance.

2.7.5 Horizontal Gene Transfer: Through procedures including conjugation, transformation, and transduction, bacteria can pick up resistance genes from other bacteria. This enables them to quickly pick up new antibiotic resistance mechanisms from other bacterial species.

2.7.6 Biofilm Formation: Like numerous other bacteria, *Pantoea ananatis* has the ability to build biofilms. Bacteria are more immune system and antibiotic resistant in biofilms' protective habitat. Antibiotic penetration into a biofilm is physically constrained by its thick matrix. It's crucial to remember that the overuse and improper use of antibiotics in medical and agricultural contexts has exacerbated the natural phenomena of antibiotic resistance. Antibiotic use can favour the survival and growth of bacteria possessing resistance characteristics, which can result in the growth and dissemination of antibiotic-resistant strains. To prevent the growth and spread of bacteria that are resistant to antibiotics, it is essential to use antibiotics responsibly, take infection control precautions, and conduct research on novel medicines.

CHAPTER 3

3. Materials and Methods

3.1 Sample Collection

The sample of NCCP 568 strain of *P. ananatis* has been isolated from rice leaves (super variant), which were infected with bacterial blight. The culture was collected from Pakistan, Kala Shah Kaku, rice field area.

3.2 Preliminary Identification

The preliminary identification of the specimen was done under the supervision of Dr. Iftikhar Ahmed, the National Agricultural Research Centre (NARC), and the National Culture Collection of Pakistan (NCCP) Islamabad.

3.2.1 Colony Morphology

The isolate NCCP 568 was streaked on Mannitol Salt Agar (MSA) plates; a selective media for *Pantoea* species and was incubated at 37°C. After 24 hours of incubation, the colony size, shape, colour, texture, and margin were recorded and crosschecked with Bergey's Manual of Systematic Bacteriology (Bergey's Manual® of Systematic Bacteriology, 2005).

3.2.2 Cell Morphology

Gram staining was performed to know about the cell morphology. For this, a smear of a pure and fresh culture was prepared on a glass slide. The slide was then stained with primary dye (crystal violet) for two minutes, followed by staining with iodine solution for about two minutes. The slide was flooded with water to remove the excessive stain and purple-stained cells were decolorized with 70% ethanol for about 1 minute followed by staining with a secondary dye (safranin) for 45 seconds. Finally, the slide was washed, air dried, and examined under the microscope (Ahmed et al., 2014).

3.3 Biochemical Characterization

Following biochemical tests were performed to phenotypically characterize the isolate NCCP568 and to distinguish it from other bacterial strains.

3.3.1 Catalase Test and Lactose Fermentation Identification

Bacteria use catalase enzyme to protect themself from the harms of oxidizing agents like H_2O_2. Catalase has the ability to catalyze and covert damaging hydrogen peroxide into O_2 and H_2O. A fresh colony of the isolate NCCP 568 was placed on a slide using a sterilized loop and 3% of H_2O_2 was applied to observe the bubble formation (catalase positive).

Moreover, the colony colour change was also observed during colony morphology testing which suggests lactose fermentation identification is positive.

3.4 Antibiotics Susceptibility Assay

The strain NCCP 568 was tested against 23 antibiotics: ofloxacin (5 µg), Bacitracin (10 µg), amoxicillin (30 µg), ciprofloxacin (5 µg), Rifampin (5 µg), Clindamycin (2 µg), Cefotaxime (30 µg), Septran (Trimethoprim 1.25 µg, sulphamethoxzale 23.75 µg), tetracycline (30 µg), cephalothin (30 µg), Cefpodoxime (30 µg), imipenem (10 µg), Ofloxacin (5 µg), cefaclor (30 µg), Kanamycin (30 µg), ampicillin (30 µg), amikacin (30 µg), sulbactam/ Cefoperazone (150 µg), Colistin (10 µg), Chloramphenicol (30 µg), Ceftazidime (30 µg), streptomycin (10 µg), Erythromycin (15 µg). The antibiotics susceptibility assay was performed using the Kirby-Bauer method. Briefly, the bacterial culture at a turbidity of 0.5 McFarland was prepared from an overnight culture by mixing pure colony in 1 ml of normal saline. 50µl of bacterial suspension was then poured and spread over Mueller-Hinton Agar (MHA) media plates using a glass spreader to make lawn and 4-5 antibiotic discs were placed on each plate. After 24 hours of incubation at 37°C zone of inhibition was recorded and examined as per the guidelines of the Clinical and Laboratory Standards Institute (‘CLSI’, 2019).

3.5 Glycerol Stock Preparation

Aqueous solution of glycerol acts as a cryo-protectant and is ideal for preserving microbial culture at low temperatures. About 1 ml of broth from overnight fresh culture was taken and centrifuged for 5 minutes at 4500 RMP; the centrifugation step was repeated in order to get a concentrated pallet. The thick pallet was dissolved in LB broth from which 1ml was mixed in 0.5ml of 80% sterile glycerol. The cryovials were then gently vortexed and frozen using liquid nitrogen before being transferred to a -80°C freezer.

3.6 Molecular Identification

3.6.1 DNA Extraction

DNA was extracted from the pure culture using the PureLink® Genomic DNA Mini Kit at NARC Lab using a self-optimized manufacturer's protocol. 400 ml of 1M Tris HCL, 40 ml of 0.5M EDTA, 240 ml of Triton X-100, and 20 mg/ml fresh lysozyme were used to make a self-made lysozyme digesting buffer. By adding distilled water, the lysozyme digestion buffer's capacity was increased to 20 ml. The pellet from a fresh broth culture was treated in lysozyme buffer for an hour at 37°C, then 200 l of genomic lysis/binding buffer and 20 l of proteinase K were added, and the mixture was again incubated for 40 minutes at 55 °C. 200 l of 96% pure ethanol were added after incubation to eliminate the contaminants. After filtering the column with Tris buffer, DNA was then eluted and saved.

3.6.2 DNA Quantification and Integration

Extracted DNA was quantified using a Qubit 2.0 fluorometer. The Integrity and quality of extracted DNA were checked via Gel Electrophoresis using 0.75% agarose gel with a 1kb ladder.

3.6.3 Amplification

The amplification process is done through PCR using universal forward and reverse primers

- Forward primer : 9F: 5'-GAGTTTGATCCTGGCTCAG-3'
- Reverse primer: 1510R: 5'-GGCTACCTTGTTACGA-3'

Table 3: Composition of 25 µL cyber green master mix

Reagents	Volume (µL)	Concentration (X)
Cyber Green Master Mix	12.5	
Reverse Primer	1.5	1ng
Forward Primer	1.5	1ng
Nuclease-Free Water	8.5	
Template DNA	1	

The typical procedure outlined by Javed et al. (2017) was adhered to. To identify the nomenclature of isolates, the sequences of closely related species were downloaded from the ExTaxon service (https://eztaxon-e.ezbiocloud.net/dashboard). The MEGA 7 software's maximum likelihood (ML) technique was used to build the polygenetic tree. The bootstrapping of 1000 replicates was used to evaluate the nodes' stability. Each strain's DNA accession numbers were received from the National Centre for Biotechnology Information (NCBI).

3.7 Whole Genome Sequencing

The amplified PCR product was sequenced using universal 16S rRNA sequencing primers at Macrogen, Korea (Seoul, Korea).

3.8 Reference Based Assembly

P. ananatis Reference strain PA13 was retrieved from NCBI. The sequence files were aligned using the move contigs tool in Mauve and finally, a multi-fasta sequence of *P.*

ananatis NCCP568 was saved (Darling et al., 2004). The newly generated multi-fasta. FNA file of *P. ananatis* NCCP568 contigs were in the specified order as that of the reference. The aligned multi-fasta.fna file was opened in Artemis and a single fasta file was created with the write-all bases option (Budin, 2015).

3.9 Retrieval of Related Strains of *P. ananatis*

Whole genome sequences of 11 closely related strains of *P. ananatis* were retrieved from the NCBI data base along with other general information of all those strains. The whole genome sequence of these strains would be further used in comparative study for this research.

3.10 Identification of Antibiotic Resistance Genes

To know about the genetic bases of resistance in the *P. ananatis* NCCP568, resistance genes in the genome were identified using the Resistance Gene Identifier (RGI) tool at the Comprehensive Antibiotic Resistance Database (CARD) server (McArthur et al., 2013) and ResFinder (Zankari et al., 2012) at CGE server. The selection criteria for CARD database were perfect and strict. ResFinder upper threshold of 90% with a minimum length of 60% was used for the identification of acquired antimicrobial resistance genes.

3.11 Comparative Analysis of Resistance Genes

The predicted antimicrobial resistance genes in *P. ananatis* NCCP568 were compared with the other *P. ananatis* complete genomes available in the NCBI database in order to compare the resistance profile and to identify any novel resistance genes in local strain NCCP568.

3.12 Identification of Mobile Genetic Elements (MGEs)

Mobile Genetic Elements (MGEs) are DNA fragments that encode proteins associated with resistivity, pathogenicity, and enzymes that mediate their transfer and integration into another DNA. These include plasmids, bacteriophages, pathogenicity islands, and transposons

Malachowa and Deleo, 2010). To predict IME in our local strain ICEberg 2.0 (Bi et al., 2012) was used with default parameters. This is an online IME analysis tool and gives comprehensive information about bacterial integrative and conjugative elements (ICEs) (Bi et al., 2012).

3.13 Identification and Comparison of Prophage Sequences

Bacterial strains can also acquire antimicrobial resistance genes from prophages that can integrate and become part of a bacterial genome. PHASTER (Arndt et al., 2016) an online tool http://phast.wishartlab.com/, was used to predict the insertion of prophages in the genome of *P. ananatis* NCCP568. The predicted phage sequences were retrieved from the genome and checked for the presence of virulence factors and antibiotic-resistance genes.

3.14 Identification and Comparison of Plasmid

The whole genome sequence of *P. ananatis* NCCP568 was blasted with the plasmids of the reference genome and 11 other related *P. ananatis* species using the NCBI blast tool (Ye et al., 2013).

3.15 Identification of insertion sequences

Genome plasticity can also be studied by the presence of insertional sequences that become the part of mobilome. These insertional sequences can be transferred from one locus to another and within cells and mediate genome diversification and dynamicity. Insertional sequences in the genome of *P. ananatis* NCCP568 were studied by using ISfinder (Siguier, 2006).

3.16 Identification and Comparison of Virulence Factors

The gene products that cause the pathogen to survive and cause infection in the host are termed virulence factors. Prediction of these factors can help in understanding how virulent

the pathogen is, so the genome of *P. ananatis* NCCP568 was comparatively analyzed by performing BLASTn search against VFDB (virulence factor database) (Chen et al., 2005) at (http://www.mgc.ac.cn/VFs/). The Predicted Virulence factors were then compared with the other available complete genomes of *P. ananatis*.

3.17 Identification of CRISPER-Cas System

CRISPR (clustered regularly interspaced short palindromic repeats) arrays and their associated proteins (Cas) in bacteria confer adaptive immunity against mobile genetic elements, such as phages and/or plasmids. CRISPR-Cas in the *P. ananatis* NCCP568 was identified by using CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr), which allows both the identification of Cas proteins and CRISPR arrays in the input genome sequence (Couvin et al., 2018).

3.18 Whole Genome Sequence Comparison and Visualization

The whole genome sequence of NCCP568 underwent BLAST with reference genome PA13 and the resultant genome was visualized. Furthermore, the over lapping genes of PA13, NCCP568, and JT8-6 are visualized on ORTHOVENN keeping the setting at default.

3.19 Phylogenetic Analysis Based on Pan and Core Genome

The pan and core genome of all the genomes of *P. ananatis* were estimated using Bacterial Pan Genome Analysis BPGA software at default parameters (50% sequence identity threshold). The orthologous proteins were clustered using the USEARCH clustering algorithm and protein alignment files used for pan-genome and core-genome construction.

3.20 Pan and Core Genome Analysis

The whole genome sequence of NCCP568 along with the related strain of *P. ananatis* was added to the BPGA pipeline. It gave an in-depth analysis of the core and pan-genome of the species. Default parameters were set (50% sequence identity threshold).

CHAPTER 4

4. Results and Discussion

4.1 Characterization of Collected Strain NCCP 568

The strain NCCP 568 was able to grow on differential growth media Mannitol Salt Agar (MSA) plates and visible colonies were observed after 24 – 72 hours of incubation at 37˚C. The isolated colonies were further identified based on morphological and biochemical characteristics. The colony morphology was small, smooth, entire, convex, opaque, and yellow in color. The colonies were gram-negative and biochemical tests showed that the colonies were catalase positive and coagulase negative.

Before NCCP 568 no strain of *P. ananatis* from Pakistan has been sequenced or analyzed. More than 12 strains from China and some from Korea are reported to have similar characteristics (Arayaskul et al., 2020).

4.2 Retrieval of Related Strains of *P. ananatis*

P. ananatis strains were selected. Out of which, PA13 is a representative strain and 10 others are strains that have specifically rice or plant hosts (Abdel-Gaied et al., 2022). The NCCP 568 strain was collected from Kala Shah Kaku, Punjab, Pakistan. General information about each strain is listed in table 4.

It can be observed that NCCP 568 has the highest protein count of 4764 as compared to the related strains. Whereas, the genome length is slightly reduced to 4.688 Mbp.

Table 4: Basic information on the local strain of *P. ananatis* NCCP 568 and related *P. ananatis* strains

Strains of *P. ananatis*		**Host**	**GC %**	**Total length (Mb)**	**Protein count**	**Source**
	NCCP568	rice	53.49	4.685862	4764	Pakistan, 2022
Ref. sequence	PA13	plant	53.6192	4.86713	4423	Korea, 2012
	JT1-188	rice	53.3749	5.08733	4563	China, 2022
	R100	rice	53.597	4.85786	4290	China, 2016
	JT8-6	rice	53.3233	4.96637	4410	Chine 2022
	YJ76	rice	53.6389	5.14648	4582	China, 2017
	NN08200	plant	53.7703	5.17664	4645	China, 2019
	JBR-LB3-16	plant	53.4897	4.91503	4362	Korea, 2020
	FDAARGOS_680	plant	52.958	5.27924	4731	USA, 2019
	AJ13355	plant	53.6813	4.87728	4246	Japan, 2011
	LMG 5342	plant	53.2829	4.90814	4353	Georgia, 2012
	TZ39	plant	53.3326	4.89569	4318	China, 2021

4.3 Phenotypic Resistance Profile

The phenotypic resistance profile showed that NCCP 568 is resistance against ofloxacin (5 µg), Bacitracin (10 µg), amoxicillin (30 µg), ciprofloxacin (5 µg), Rifampin (5 µg), Clindamycin (2 µg), Cefotaxime (30 µg), Septran (Trimethoprim 1.25 µg, Sulphamethoxzale 23.75 µg), Tetracycline (30 µg), Cephalothin (30 µg), Cefpodoxime (30 µg), Imipenem (10 µg), Ofloxacin (5 µg), Cefaclor (30 µg), Kanamycin (30 µg), Ampicillin (30 µg), Amikacin (30 µg), Sulbactam/ Cefoperazone (150 µg), Colistin (10 µg), Chloramphenicol (30 µg), Ceftazidime (30 µg), Streptomycin (10 µg), Erythromycin (15 µg). The zone inhibition of each is given in the table 5.

The phenotypic resistance profile of NCCP 568 is similar to those of its related stains. Similar antibiotic resistance patterns can be seen in the strains from China and Korea. (Bajpai et al., 2020)

Table 5: List of antibiotics and NCCP568 resistant or susceptible to those antibiotics

Antibiotics	**Abbreviation**	**Zone (mm)**	**Resistant**	**susceptible**
Ofloxacin (5 µg)	OFX	16		yes
Amoxicillin (30 µg)	AMX	18	yes	
Bacitracin (10 µg)	B			yes
Ciprofloxacin (5 µg)	CIP	25		yes
Rifampin (5 µg)	RA		yes	
Clindamycin (2 µg)	CLI		yes	
Cefotaxime (30 µg)	CTX	15	yes	
Septran (Trimethoprim 1.25 µg, sulphamethoxzale 23.75 µg)	SXT	22	yes	
Tetracycline (30 µg)	TE	24		yes
Cephalothin (30 µg)	CT	13	yes	
Cefpodoxime (30 µg)	CPD	21	yes	
Imipenem (10 µg)	IPM	16	yes	
Ofloxacin (5 µg)	OFX	16		yes
Cefaclor (30 µg)	CEC	18	yes	
Kanamycin (30 µg)	K	13		yes
Ampicillin (30 µg)	AMP	17	yes	
Amikacin (30 µg)	AK	10		yes
Sulbactam/ Cefoperazone (150 µg)	S	11	yes	
Colistin (10 µg),	E	17	yes	
Chloramphenicol (30 µg)	CHL	12	yes	
Ceftazidime (30 µg)	CAZ	15	yes	
Streptomycin (10 µg)	STR	21	yes	
Erythromycin (15 µg)	ERY	24	yes	

4.4 Whole Genome Sequence, Assembly and Annotation

Pantoea sp. NCCP-568 was assembled using auto stofware. There were 21 contigs, an estimated genome length of 4,685,862 bp, and an average G+C content of 53.49%. The N50 length, which is defined as the shortest sequence length at 50% of the genome, is 592,727 bp. The L50 count, which is defined as the smallest number of contigs whose length sum produces N50, is 3.

Table 6: NCCP 568 sequence Assembly details

Contigs	21
GC Content	53.49
Plasmids	0
Contig L50	3
Genome Length	4,685,862 bp
Contig N50	592,727
Chromosomes	0

The resultant sequence is aligned with reference strain PA13, having a 4.9 Mb genome size, available at NCBI under the accession number GCA_000233595.1, using Mauve software. A single assembled FASTA file was generated.

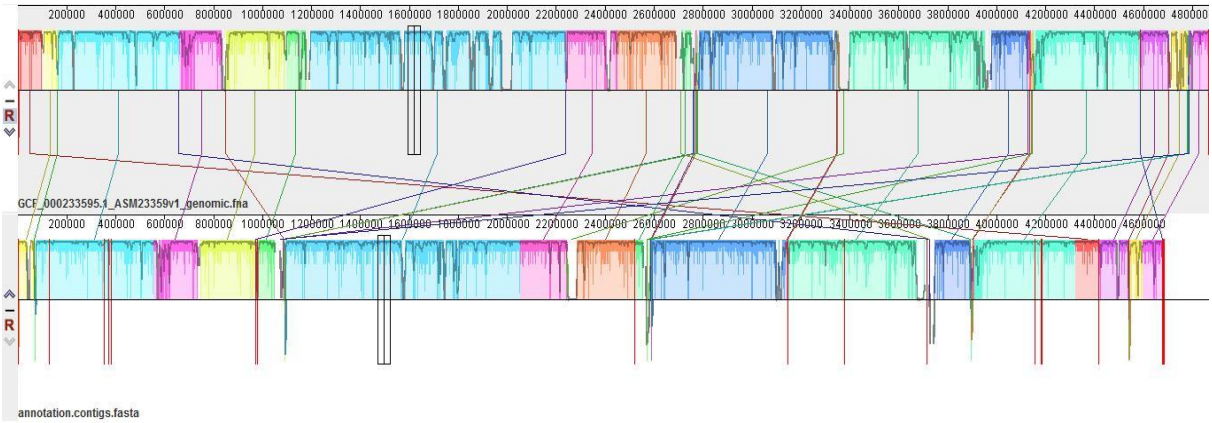

Figure4 : MAUVE Sequence Alignment result of NCCP568 (below) with the reference sequence of PA13 (above)

This genome has 4,764 protein-Coding Sequences (CDS), 66 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes. The annotated features are summarized in Table. The annotated gene was further visualized in PATRIC to map resistance and virulence genes.

Table 7: Summarized annotated features of NCCP 568

CDS	4,764
tRNA	66
rRNA	2
Partial CDS	0
Miscellaneous RNA	0
Repeat Regions	0

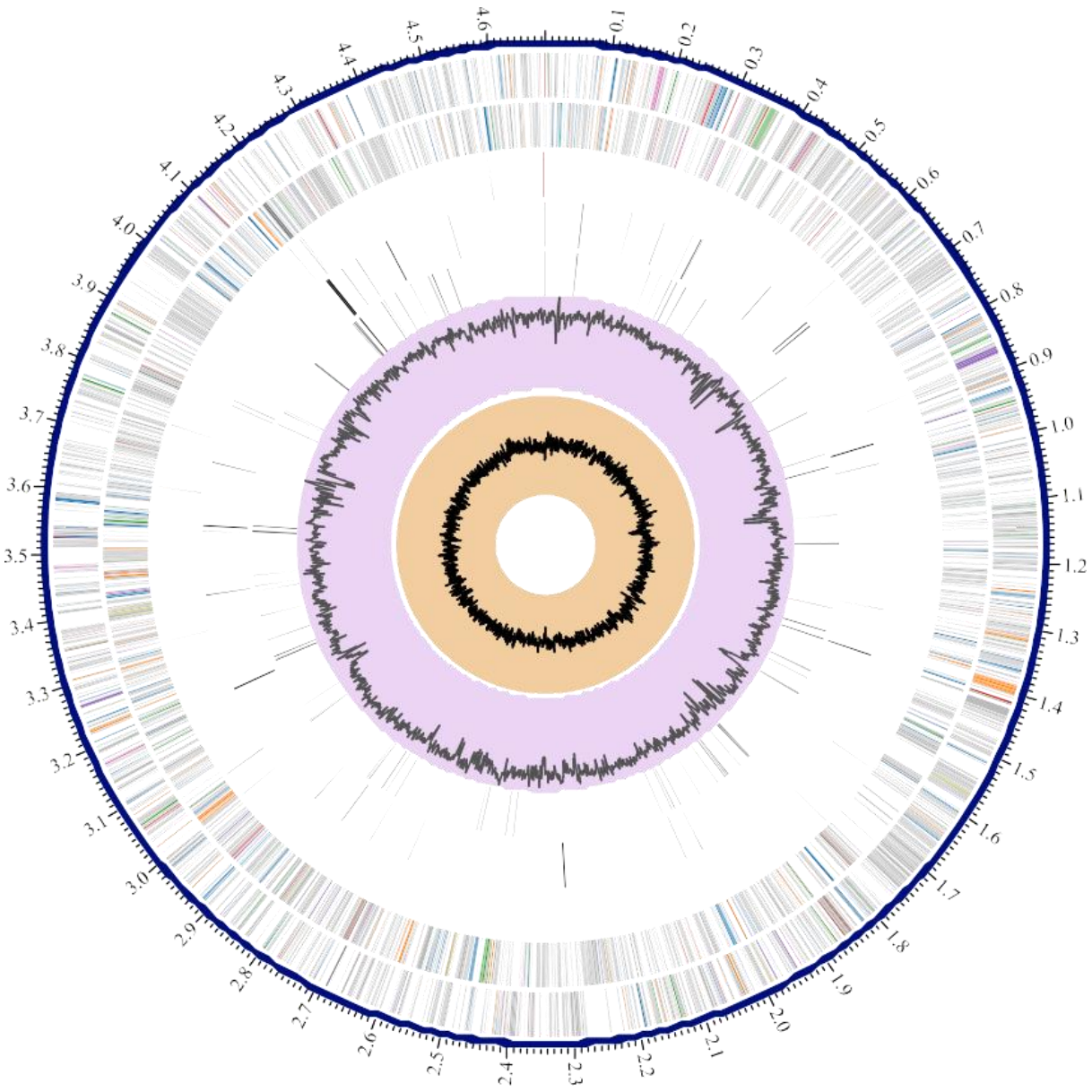

Figure 5: Genomic Visualization on PATRIC

A circular graphical display of the distribution of the genome annotations is provided (Figure 5). This includes, from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content, and GC skew. The colors of the CDS on the forward and reverse strands indicate the subsystem that these genes belong to.

4.5 Insertion sequence identification and comparison

More than 8 insertion sequence families were identified in the local stain of *P. ananatis* NCCP568 including IS3, IS30, IS110, ISNCY, Tn3, IS66, and IS4. Other identified families had e-value of more than 0.05. The Large number of insertion sequences probably facilitated frequent genomic reorganization in NCCP 568 and might led to phenotypic diversification. Identified insertion sequences suggest transposition within the genome of NCCP 568 strain. (Arayaskul et al., 2020)

Table 8: List of identified insertion sequences

IS3	IS30	IS110	ISNCY	Tn3	IS66	IS4
Escherichia coli	*Citrobacter freundii*	*Klebsiella quasipneumoniae*	*Raoultella ornithinolytica*	*Salmonella enterica*	*Escherichia coli*	*Pectobacterium carotovorum*
Enterobacter cloacae	*Dickeya dadantii*	.	*Citrobacter freundii*	*Pseudomonas aeruginosa*	*Shigella dysenteriae*	*Dickeya dadantii*
Pantoea agglomerans	.	.	.	.	*Klebsiella pneumoniae*	*Citrobacter rodentium*
Erwinia amylovora	.	.	.	.	*Klebsiella oxytoca*	.
Salmonella enteritidis	.	.	.	.	*Shigella flexneri*	.
Enterobacter agglomerans	.	.	.	.	.	.
Salmonella enterica	.	.	.	.	.	.
Klebsiella pneumoniae	.	.	.	.	.	.
Pseudomonas entomophila	.	.	.	.	.	.

4.6 Prophage Identification:

One intact phage region "region 5" of 48.6 kb was identified, which is a close match to Phage Klebsiella, with a GC% of 50.6, located in the region 2239334-2287945 on the whole genome. Regions 1, 3, and 4 were incomplete phage regions and region 2 was a questionable match. Hence, regions 1, 2, 3, and 4 are not considered intact prophage regions. (Figure 6)

The detailed structure of PHAGE_Klebsi_phiKO2 can be seen below in figure 7. Bacteria are able to acquire AMR through these phage regions. PHAGE_Klebsi has a prominent role in AMR. (Carr et al., 2010)

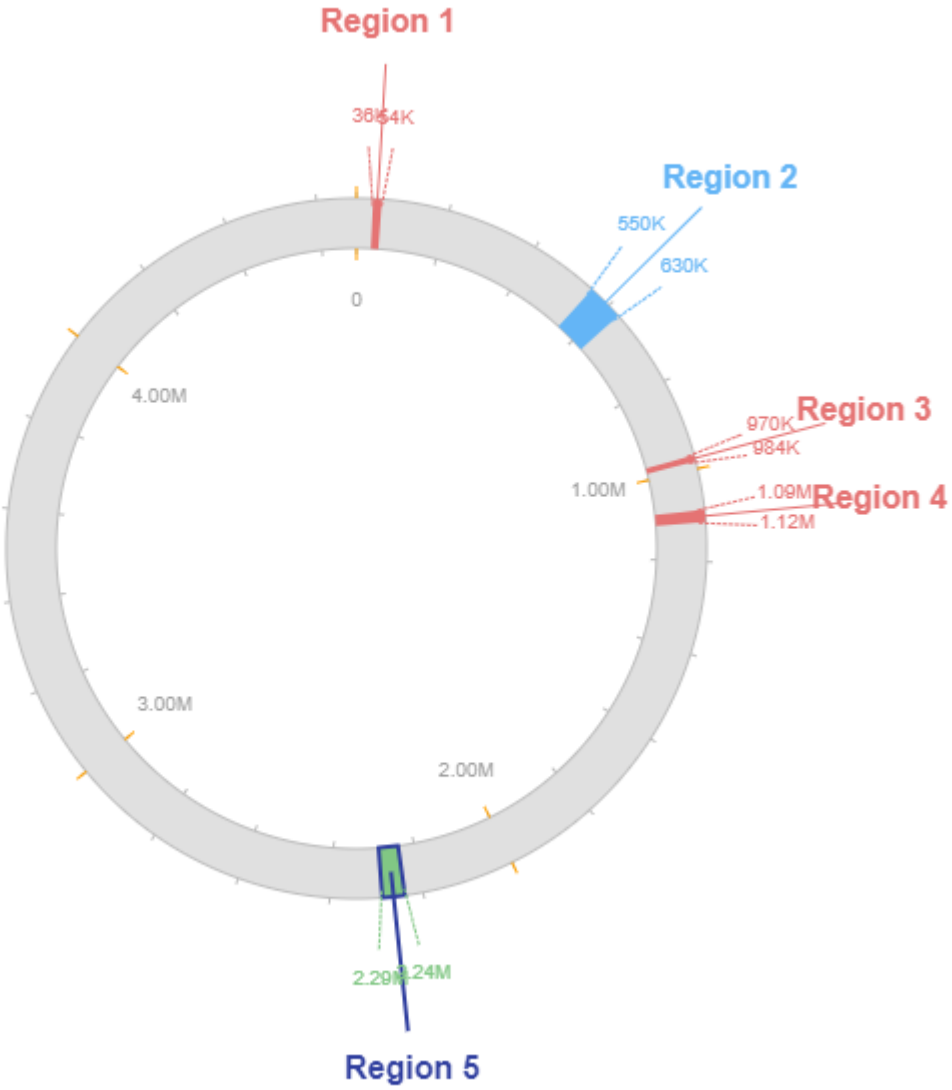

Figure 6 : Prophage regions identified on the circular genome of NCCP568

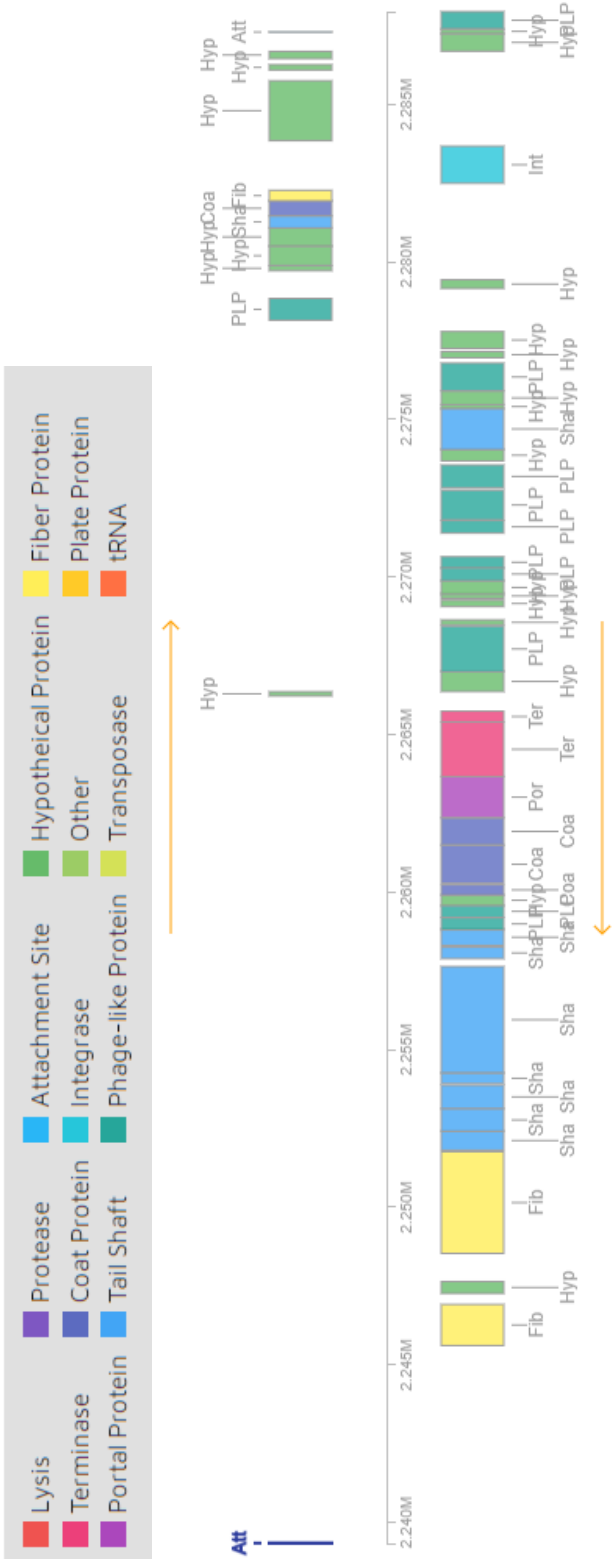

Figure7 : The detailed structure of PHAGE_Klebsi_phiKO2

4.7 Antibiotic resistance gene identification and comparison:

ResFinder and CARD identified several resistant genes in *P. ananatis* NCCP568 including vanG, ArnT, PBP3 (*Haemophilus influenza),* gyrB (*Morganella morganii),* CRP, rsmA, KpnH (*Klebsiella pneumonia)* and adeF genes. These genes are known for conferring resistance against *vancomycin, colistin* A, *colistin* B, beta-lactam antibiotics (ceftriaxone, ampicillin, cefaclor, cefotaxime, cefditoren, cefdinir), fluoroquinolones (enoxacin, ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, trovafloxacin, grepafloxacin, sparfloxacin, pefloxacin), erythromycin, cloxacillin, oxacillin, norfloxacin, azithromycin, imipenem, polymyxin B, polymyxin B1, B2, B3, B4 and tetracycline. Two prominent resistance mechanisms shown by the given genes are antibiotic target alteration and antibiotic efflux.

Literature proves that the antibiotic efflux resistance mechanism is commonly used by most of the *P. ananatis* strain but antibiotic target alteration is more specific to the NCCP 568 strain. (Lv et al., 2022)

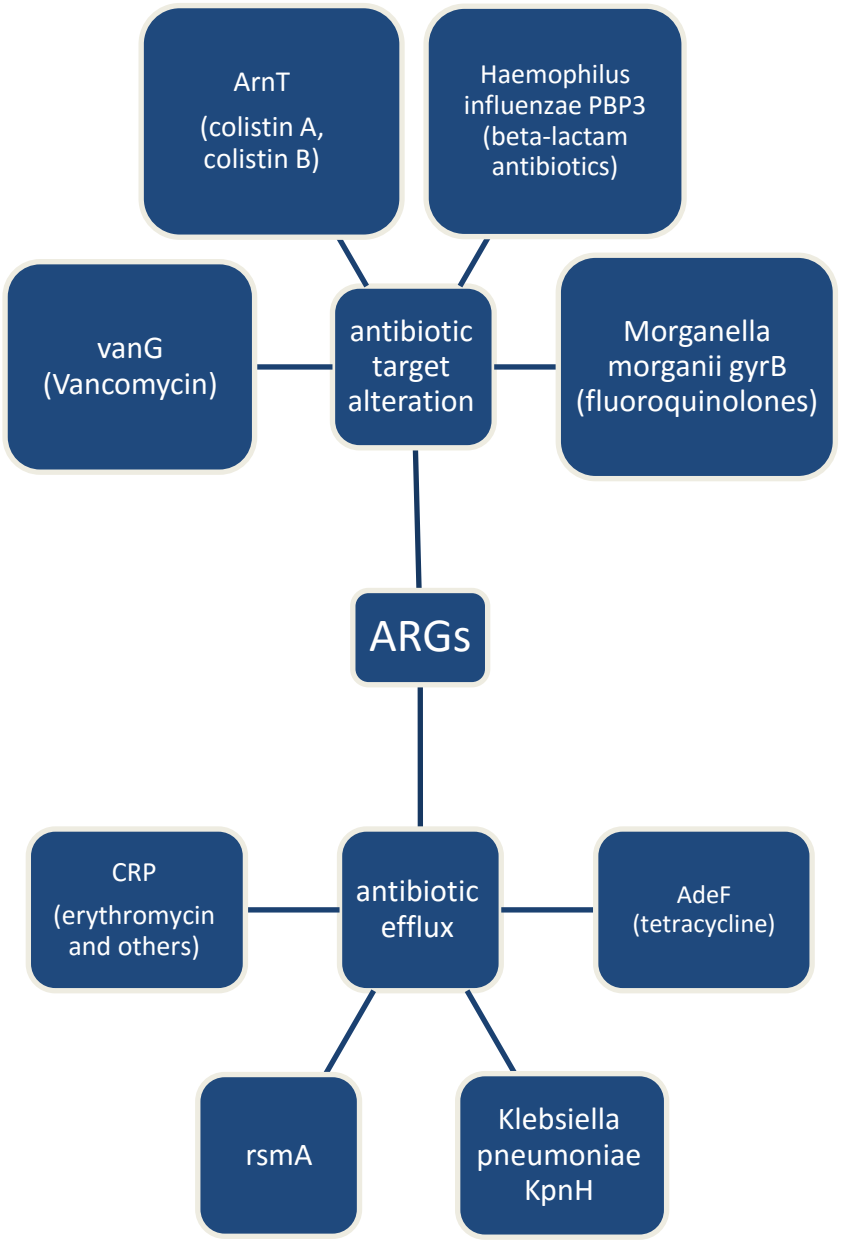

Figure 8: Resistant genes of NCCP 568 identified using CARD and ResFinder

4.8 Identification of CRISPER-Cas Region:

After running the whole genome of *P. ananatis* NCCP568 in CRISPER-Cas finder, no CRISPER-Cas regions were identified.

4.9 Phylogenetic Analysis:

Phylogenetic analysis using pan and core genes from all available genomes provided a means for estimating evolutionary relationships between the different strains of *P. ananatis*. The evolutionary relationship of NCCP568 with all available strains of *P. ananatis* showed that *P. ananatis* NCCP568 shares clade with the strain FDAARGOS, JT1-188, TZ39, JT8-6 and Lstri.

Further, a strain PA13, the reference strain was found to be part of a different clad along with strains LMG5342, R100, and FU01. The uniformity depicted from both these analyses supports the notion that these different approaches were not only comparable but also provided meaningful and consistent results which indicate the reliability of the approaches used for elucidating evolutionary relationships between the different strains of *P. ananatis*. There were some slight differences which can be seen. Core Phylogeny shows that NCCP568 is more closely related to JT1-188 whereas pan phylogeny shows that NCCP568 is closely related to JT8-6 and Lstri strains (Hara et al., 2012).

4.9.1 Pan phylogeny of NCCP 568 with Reference to Related *Pantoea* Strains

The tree was constructed using the neighbour joining method and 500 bootstrap value.

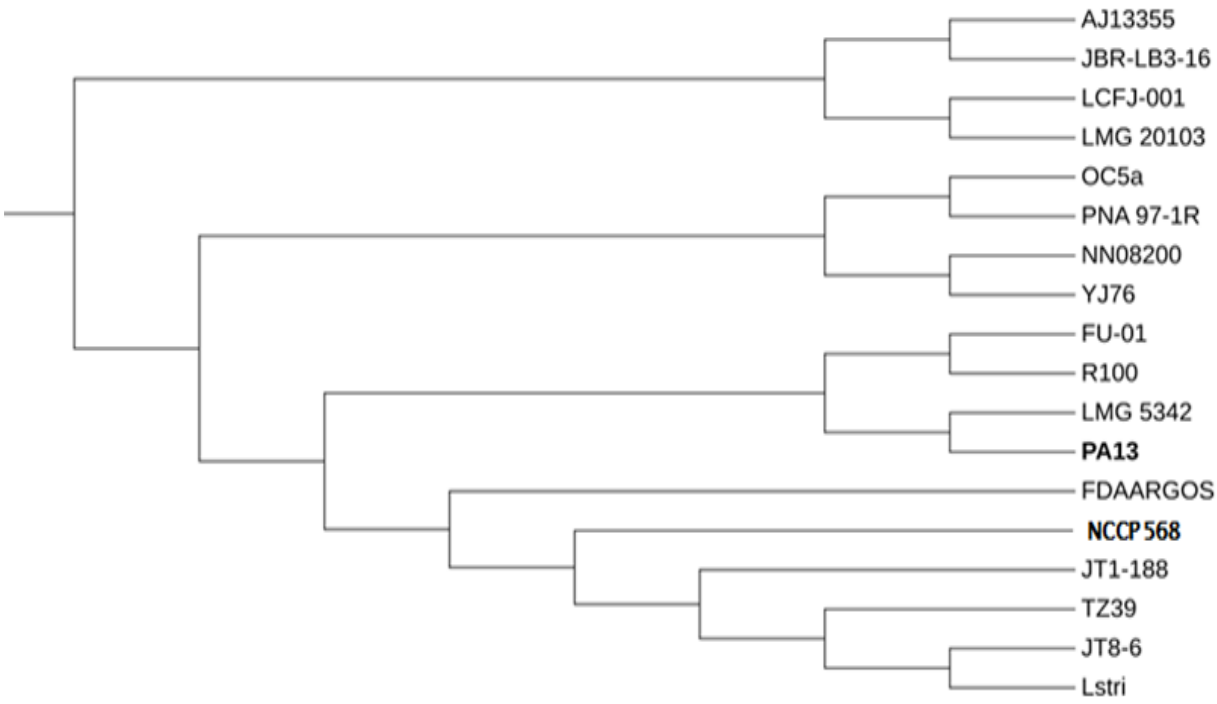

Figure 9: Pan Phylogeny

4.9.2 Core phylogeny of NCCP 568 with Reference to Related *Pantoea* Strains

The tree was constructed using the neighbour joining method and 500 bootstrap value.

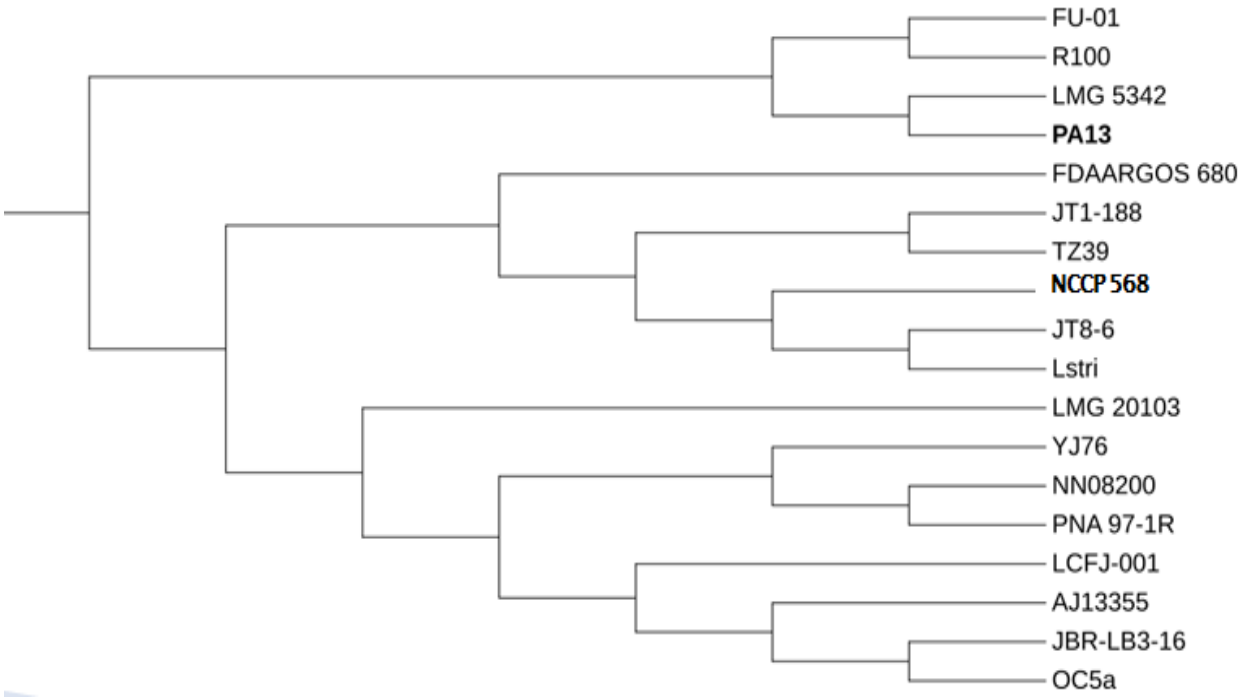

Figure 10: Core Phylogeny

4.10 Whole Genome Sequence Comparison and Visualization

Genome visualization is depicted in figure 11. The Blast with representative *P. ananatis* strain PA13 is shown in the outer most ring. Adjoining to this are the coding regions of local strain NCCP568. The coding regions are intersected with blue and red lines of non-coding rRNA and tRNA. Then the ring representing the GC% represented in green is present.

ORTHOVENN result shows the Venn diagram representation of the number of overlapping Core genes and the unique accessory genes of Local strain NCCP568, Representative strain PA13, and closely related *P. ananatis* strain JT8-6 from China origin (figure 12). This shows that the local strain has a total of 3784 core genes, more than 135 softcore genes, and 31 accessory genes. If compared with the literature we can observe that a variety of new genes have been added to the pan-genome of NCCP 568 (Bajpai et al., 2020).

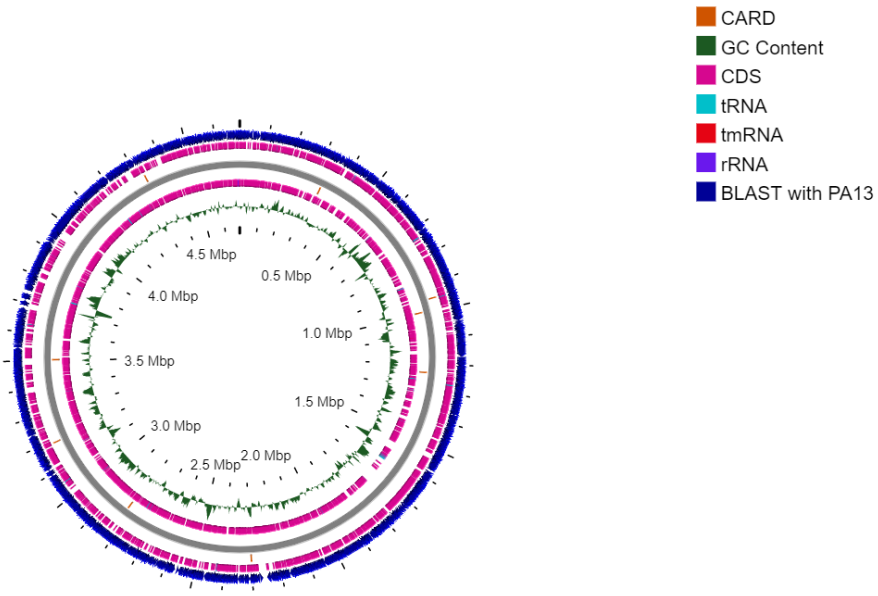

Figure 11: Whole genome comparison with PA13 using BLAST and Genome visualization showing CDCs, GC content, tRNA, rRNA, and the resistance genes CARD

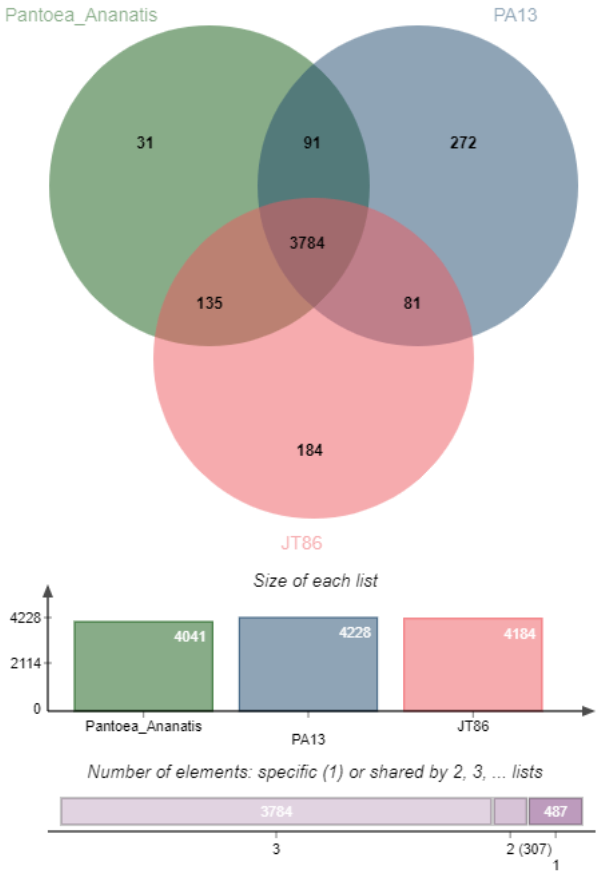

Figure12 : Venn daigram of genes of NCCP568, PA13 and JT8-6

4.11 Pan Core Genome Analysis

The graph shows the comparative relation of pan and core genes (figure 13). The core genes depict a slightly straight line, showing the consistent number of gene families throughout the genome. Pan genome, on the other hand, shows a gradual increase with gene families along the genome as more and more number of accessory genes are added to the pool. The arch of the pan-genome shows how diverse *P. ananatis* is and suggests an open- pan genome. It can be seen that a variety of new genes are added to the pan genome compared to the existing strains of *P. ananatis* (Agarwal et al., 2021).

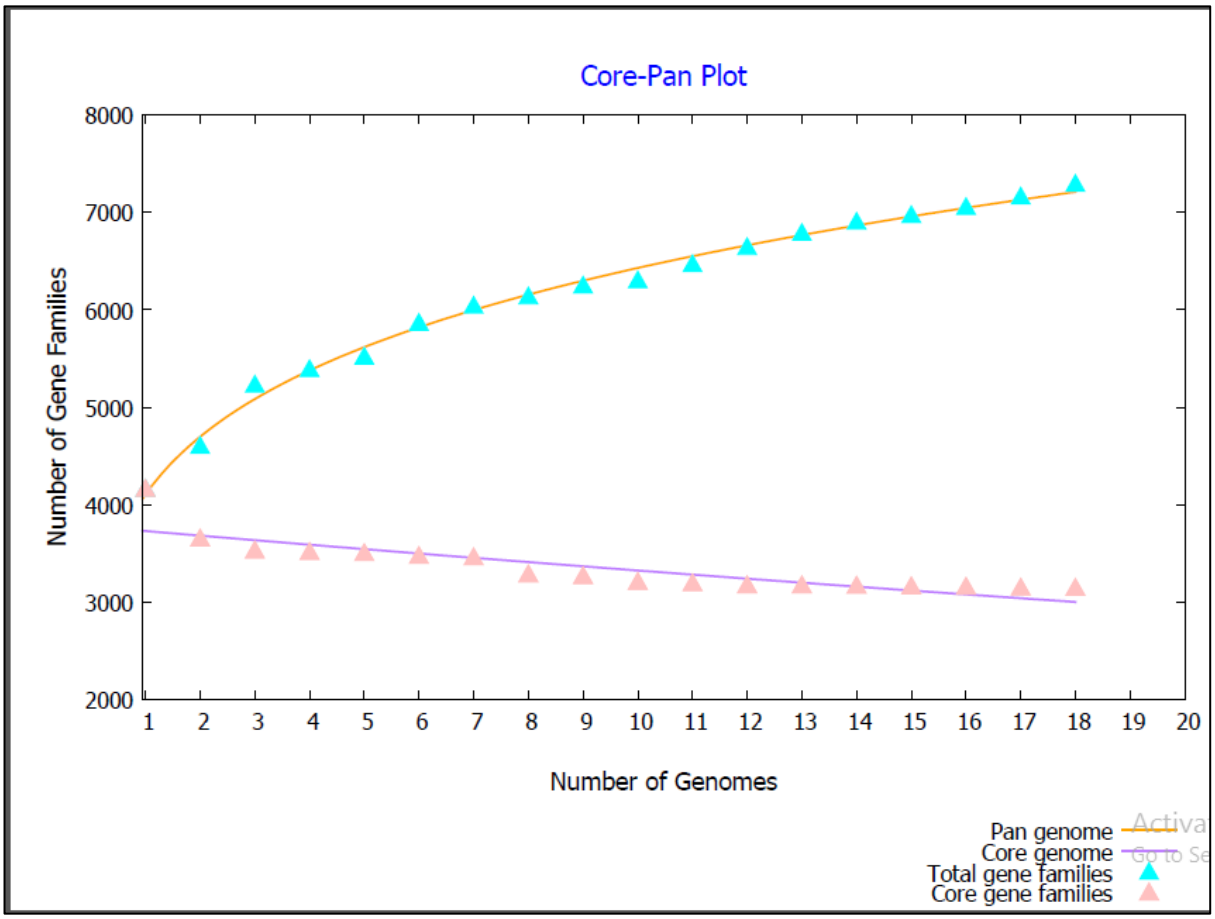

Figure13 : Pan Core genome analysis graph

CHAPTER 5

5.1 Conclusions

After a comprehensive comparative genomic analysis on *P. ananatis* NCCP 568, it can be concluded that a variety of new genes has been added to the pan-genome of NCCP 568. This proves that *P. ananatis* is still evolving with an open-pan genome. The local stain NCCP 568 has a total of 3784 core genes and 31 accessory genes. It has also been observed that some of the core genes have been reduced compared to the reference stain of *P. ananatis* PA13. Moreover, NCCP 568 has no plasmid and zero chromosomes in its DNA with 4764 CDS and a genome length of 4.685 Mbp.

The whole genome sequence and comparative genome analysis of a local *P. ananatis* strain NCCP 568 show relevantly more resistance determinants and virulence genes compared to other complete genomes of *P. ananatis*. It shows multi-drug resistance to a variety of antibiotic groups including vancomycin, colistin A, colistin B, beta-lactam antibiotics, fluoroquinolones, erythromycin, cloxacillin, oxacillin, norfloxacin, azithromycin, imipenem, polymyxin B, polymyxin and tetracycline.

It has been reported that similar strains located in different geographic locations sometimes depict different phenotypic characteristics due to the ability of *P. ananatis* to adapt to its environment with slight genetic modifications. Hence it is crucial to sequence and study the genetics of local strains. No local strain of *P. ananatis* has been sequenced or published before NCCP 568. As a plant pathogen and a growth promoter in various agriculturally important plants, the genomic analysis of strain NCCP 568 is of significance to an agricultural country like Pakistan.

5.2. Recommendations and Future Prospects

Understanding *P. ananatis* biology, toxicity, and possible uses in the environment and biotechnology depends heavily on genetic research. Researchers can acquire insights into the molecular interactions between the bacteria and its host through genetic studies that clarify the virulence factors and processes used by *P. ananatis*, ultimately leading to the creation of successful disease management solutions. *P. ananatis* has the potential to be used in biotechnology. Genetic research can reveal the genes that produce useful secondary metabolites, enzymes, and other bioactive substances. These genetic insights can help with process optimization, yield improvement, and efficiency enhancement of biotechnological applications including pharmaceutical development, enzyme synthesis, and biocatalysis. Researchers can change *P. ananatis* DNA and other genetic components by understanding its genetics.

The unique nature of *P. ananatis* makes it a perfect potential biotechnological tool to be utilized in agriculture and other environmental sectors. Keeping this view in mind following are some recommendations and future prospects:

1. NCCP 568 was the first local stain of *P. ananatis* to be sequenced and genetically studied. More local strains of *P. ananatis* need to be identified, sequenced, and genetically analyzed to get a better understanding of the local strains and their genetic variations.
2. Pakistan being an agriculture country is facing serious threats from diseases caused by *P. ananatis*. This can be countered genetically and more studies in this domain are required
3. *P. ananatis* has shown potential in agriculture for its ability to promote plant growth, control plant diseases, and enhance crop yield. It can act as a bio-control agent against various plant pathogens and also has the ability to produce plant growth-promoting substances and show antifungal activities.

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